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Purification of Catalase Enzyme from *Nostoc* and its Physiochemical Properties

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Abstract: Catalase(CAT) is a tetrameric enzyme consisting of four identical tetrahedrally arranged that contains a single ferriprotoporphyrin group per subunit. CAT reacts very efficiently with H_2O_2 to form water and molecular oxygen; and with H donors with peroxidase activity. The aim of this research is to purify the enzyme catalase from fresh water algae *Nostoc* through Sephadox G-75 column, its molecular weight was determined by using polyacrylamide gel electrophoresis and the catalase enzyme stability were observed at various temperature and different pH conditions. Under denaturing conditions, polyacrylamide gel electrophoresis revealed dissociation of a major component of molecular weight 34kDa, which constituted 90% of the total protein of the stained gel, suggesting that the native enzyme is tetrameric. The optimum temperature and pH for the purified enzyme catalase from *Nostoc* were confirmed at 30°C and pH 7.5.

Key words: Tetrameric · Algae · Sephadox G-75 and polyacrylamide

INTRODUCTION

Enzymes are very important molecules which found in all the living cells. Enzymes generally act as catalysts that increase the speed or rate at which substances in a cell get converted into other substances. Enzymes are the biocatalysts produced by living cells and catalyze numerous metabolic reactions occurring within the cell. They are protienaceous in nature. Enzymes influence the rate or the speed at which a biochemical reaction attains equilibrium. Various factors such as pH, temperature, ionic strength, enzyme substrate and product concentration influence the enzyme activity.As per the recommendations of the enzyme commission (IUB), enzymes are divided into six major groups on the basis of the type of the reaction they catalyze.

Enzyme class	Uses
Oxidoreductases	Catalyse oxidation reduction reactions. Ex:Lactate Dehydrogenase.
Transferases	Catalyse transfer of groups (amino, methyl, phosphoryletc) Ex: Transaminase
Hydrolases	Catalyse hydrolytic cleavage of covalent bonds. Ex: Peptidases
Lyases	Catalyse cleavage of bonds without addition of water. Ex:Aldolase
Isomerases	Catalyse intra molecular rearrangement. Ex:Phosphohexoisomerase.
Ligases	Catalyse the synthesis of a bond coupled to ATP hydrolysis. Ex:Glutamine synthase.

Catalase (E.C. 1.11.1.6; H_2O_2: H_2O_2-oxidoreductase) which belonging to the oxidoreductase family are a group of metallo enzymes with the ability to catalyze the decomposition of hydrogen peroxide into water and dioxygen. Four classes of catalases have been defined, including mono functional hem-containing catalases,

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catalase peroxidases, manganese catalases and minor catalases.CAT reacts very efficiently with H_2O_2 to form water and molecular oxygen; and with H donors (methanol, ethanol, formic acid, or phenols) with peroxidase activity:

$$2\,H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

$$ROOH + AH_2 \xrightarrow{CAT} H_2O + ROH + A$$

Catalase protects cells from hydrogen peroxide generated within them. Even though CAT is not essential for some cell types under normal conditions, it plays an important role in the acquisition of tolerance to oxidative stress in the adaptive response of cells.

Likewise, catalase has one of the highest turnover numbers of all enzymes; one catalase molecule can convert millions of molecules of hydrogen peroxide to water and oxygen each second. Catalase is a tetramer of four polypeptide chains, each over 500 amino acids long. It contains four porphyrinheme (iron) groups that allow the enzyme to react with the hydrogen peroxide. The optimum pH for human catalase is approximately 7 and has a fairly broad maximum (the rate of reaction does not change appreciably at pH between 6.8 and 7.5). The pH optimum for other catalases varies between 4 and 11 depending on the species. The optimum temperature also varies by species.

MATERIALS AND METHODS

Sample Collection and Maintenance: *Nostoc*sample was collected from Tamilnadu Agricultural University, Coimbatore, it was identified and preserved by Botanical Survey of India, Coimbatore. The *Nostoc* sample was brought to the laboratory and kept in the refrigerator for further process. The sample was inoculated into the algal culture broth (Himedia Pvt. Ltd) and kept 12 hrs dark and light cycle in laboratory condition. Strict aseptic condition maintained for the cultivation of *Nostoc*.

Extraction Preparation: The *Nostoc* was collected from the supernatant of broth and used for further enzyme purification. Ten gm of sample was crushed with 50 ml of cold 0.067M phosphate buffer (pH 7) using homogenizer to obtain fine mash and centrifuged at 10,000 rpm for 30 mins at 4°C. The supernatant was collected and used as source for further analysis.

Phytochemical Screening: Phytochemical analyses were carried out according to the methods [1] of the crude extract of algae for the identification of phytochemicals like, alkaloids, saponin, steroids, flavonoids, cardiac glycosides, cynogenic glycosides and phlobatannins.

Antioxidant, Protein and Phenolic Determination

Protein Determination: Protein content in the supernatant was estimated [2] and 0.2 ml, 0.4 ml of *Nostoc*extract was used to determine the protein content.

Total Phenolic Content: The concentration of phenolic compounds in the extract was determined [3] and results were expressed as tannic acids equivalents. The extracts were dissolved in a mixture of methanol and water (6:4 v/v). Samples (0.2 ml) were mixed with 1.0 ml of tenfold diluted Folin–Ciocalteu reagents and 0.8 ml of 7.5% sodium carbonate solution. After standing for 30 min at room temperature, the absorbance was measured at 765 nm. The estimation of phenolic compounds in the fractions was carried out in triplicate and the results were averaged.

Total Antioxidant Content [4]: An aliquot of each extracts (0.05ml) were mixed with 0.5ml of reagent (0.6M H_2SO_4, 28mM sodium phosphate and 4mM ammonium molybdate) in 1.5ml eppendorff tube. The tubes were capped and boiled in a boiling water bath at 95°C for 90min and cooled. The absorbance of each sample was measured at 695nm against blank in a spectrophotometer. A typical blank contained 0.5ml of reagent solution then 0.05ml of buffer and treated in the same manner as test. The antioxidant capacity was expressed as micromoles of ascorbic acid equivalents of antioxidant capacity.

Catalase Enzyme Assay [5]: Catalase activity was measured spectrophotometrically by monitoring the decrease in A_{240} resulting from the elimination of H_2O_2, using a Hitachi U-3210 spectrophotometer. The standard reaction mixture for the assay contained 0.067M potassium phosphate buffer (pH 7.0), 30mM $H_2O_2$and 30µl of catalase-containing solution for a total volume of 3.0 ml. On decomposition of hydrogen peroxide by catalase, the absorption decreased with time. Noted the time required for a decrease in absorbance from 0.45-0.40. The enzyme activity arrived at from this point. The extinction coefficient of hydrogen peroxide at 240 nm

was assumed to be 0.036 µm/ml and one unit (U) of catalase activity was defined as the amount of enzyme required to degrade this 1µm/ml of hydrogen peroxide.

Purification of Catalase Enzyme [6]: The supernatant brought to 45-90% saturation and kept at over night to precipitate the enzyme. The supernatant was centrifuged at 10,000rpm for 30mins at 4°C to purify the enzyme. The purified enzyme was subjected to dialysis against 50mM potassium phosphate buffer (pH 7) for changing the buffer thrice at cold condition. The dialyzed sample considered as partially purified and subjected for further purification.A column (1 by 3cm) of DEAE-cellulose equilibrated with buffer A. Thirty milliliters of the dialyzed enzyme solution was applied to the column and washed with 4 ml of buffer A. The column was eluted with distilled water. Active fraction detected by t standard assay method were collected and pooled for subsequent sephadex G-75 column.

A column of (0.5 X 5 cm) of sephadex G-75 that had been equilibrated with buffer A. The active fractions (8 ml) were diluted to 32 ml with the buffer A in order to reduce the salt concentration. The fraction was then applied to the column and washed with the 3 ml of buffer A. Elution was performed with a continuous linear gradient of 0 to 0.5M NaCl in buffer A (total volume 15 ml) and then with 3 ml of 0.5M NaCl in buffer A. The volume of 1 fraction was 1 ml. the active fractions (2 ml) were further purified by rechromatography on the same column and under elution conditions. Then the enzyme were stored at 4°C and used for the characterization of the enzyme.

Molecular Weight Determination: Purified catalase enzyme was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 12% gel as per method [7] along with standard molecular weight protein markers. The sample and marker proteins were treated with 2% SDS and 5% 2- mercaptoethanol at 100°C for 5 min just before loading. The gel was stained with Coomassie-brilliant blue R-250.

Physio-Chemical Properties of Catalase

Analysis of pH Profile of Catalase: The pH profiles for the activity of catalase was obtained using 50mM Pottasium phosphate buffer (pH 3 to pH 9). In each pH buffer (1 ml), 30µl of enzyme solution was mixed and kept for overnight at 4°C. After this period, enzyme activity of sample was analyzed under the standard assay condition.

Analysis of Thermostability of Catalase: Thirty microliters of purified catalase from *Noctoc* was placed in water bath at a temperature of 10, 20, 30, 40, 50, 60 and 70°C for periods of 60 min. Enzyme activity after treatment was analyzed under the standard assay condition.

Storage Stability: Thirty microliters purified catalase from *Noctoc* was mixed with 1ml of 50mM potassium phosphate buffer (pH 7) buffer and stored at the 4°C. Enzyme activity was analyzed every day up to 1 week in order to find out the storage stability of enzymes.

RESULTS

Phytochemical Analysis: Qualitative phytochemical analyses were performed according to the methods for the detection of alkaloids, saponin, steroids, flavonoids, cardiac glycosides, cynogenic glycosides and phlobatannins. The observed results were tabulated (Table 1). The extract of *Nostoc*contain alkaloids, saponin, steroids, flavonoids, phlobatannins, phenols and cynogenicglycoside,cardiac glycosides was absent.

Protein Estimation: Standard graph was plotted for bovine serum albumin using spectrophotometric reading at 660 nm. Total protein obtained for *Nostoc*was 30.3 mg/g (Table 2).

Total Anti Oxidant and Phenolic Content: The absorbance of the extract of each tube was measured for total anti oxidant content at 695 nm against blank it was observed that leaves contain 18.71 mg/g. The absorbance was measured at 765 nm for phenolic content. The estimation of phenolic compound in the fractions was carried out in triplicate and the results were averaged. *Nostoc* extract contained 18.56 mg/g. The results were tabulated in Table 2.

Catalase Assay: Catalase assay was performed for the mushroom extracts supernatant, enzyme activity were measured at 240nm wavelength. Time taken for the reduction of A_{240} values from 0.45-0.4 was noticed. The enzyme activity for *Nostoc* was 15.5U/ml.

Purification and Molecular Weight of Catalase from *Nostoc*: The enzyme eluted as a single species in the initial DEAE-cellulose chromatography step. Additional purification of the enzyme was achieved by gel filtration

Table 1: Phytochemical analysis of *Nostoc*

Phytochemical constituents	Observation
Alkaloids	+
Steriods	+
Saponin	+
Cynogenic glycosides	-
Cardiac glycosides	-
Flavonoids	+
Phlobatannins	+
Phenols	+

'+' – Present '-'–Absent

Table 2: Total anti oxidant and phenol content of *Nostoc*

Sample extraction	Protein content OD at 660nm (mg/g)	Anti oxidant content OD at 695 nm (mg/g)	Phenolic content OD at 765 nm (mg/g)
Nostoc	30.5	18.71	18.56

Table 3: Purification of catalase from *Nostoc*

S.No	Purification step	Total amount of protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
1	Supernatant	525	142	0.27	100	1
2	Dialysate	112	97	0.86	68	3.18
3	DEAE- cellulose	108	87	1.24	61	4.59
4	Sephadox G-75 fraction	1.12	15.5	13.8	10.9	51.11

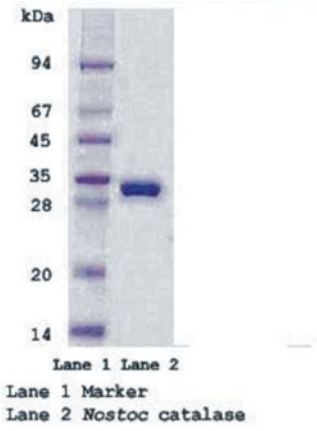

Fig. 1: Molecular mass of purified catalase

on Sephadex G-75. Table 3 summarizes the results of each step of the catalse purification. The enzyme was purified about 51.11-fold, with a final specific activity of 13.8 U/mg. The overall recovery of the purification was 10.6%. The molecular mass of purified catalase was 34 kDa (Figure 1).

Effect of pH on Catalase Activity: The pH profile on the activity of catalase enzymes from *Nostoc* was shown in Figure 2. The optimum pH for the strains of *Nostoc*was pH 7. All the catalase enzymes had a minimum activity at pH 3.5.

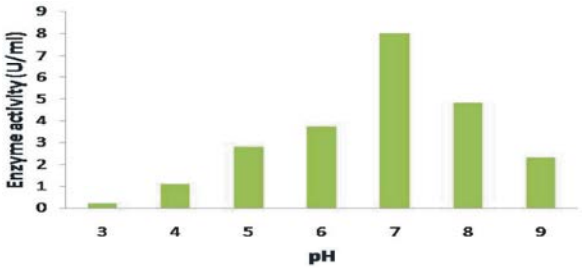

Fig. 2: Effect of pH on Catalase activity

Effect of Temperature and Storage Stability of Catalase Activity: Purified catalase from *Nostoc*was stable in 50mM potassium phosphate buffer (pH 7) for more than 4 days at 4°C, as a decrease in activity observed after 5 days (Figure 3). After being heated at 10-30°C for 60 minutes, catalase from *Nostoc*retained26.98 units/ml of the enzyme activity measured at 30°C for 60 minutes but became completely inactive after treatment between 40-60 °C for 60 minutes.

Storage stability of purified Catalase from *Nostoc*: Purified catalase from *Nostoc*was stable in potassium phosphate buffer (pH 7) for more than 4 days at 4°C, as a decrease in activity observed after 5 days (Figure 4). *Nostoc*catalase was also completely inactive under heat treatment between 40-60°C for 30 minutes. This catalase enzyme has 28.08 units/ml of activity after being heated at 10-30°Cfor 60 minutes.

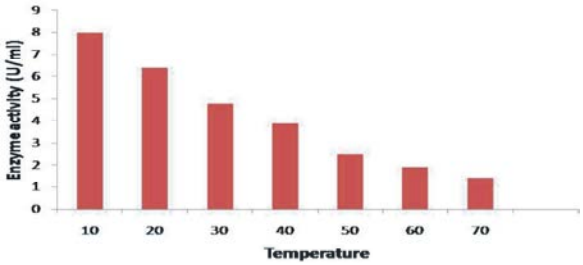

Fig. 3: Temperature stability of Catalase

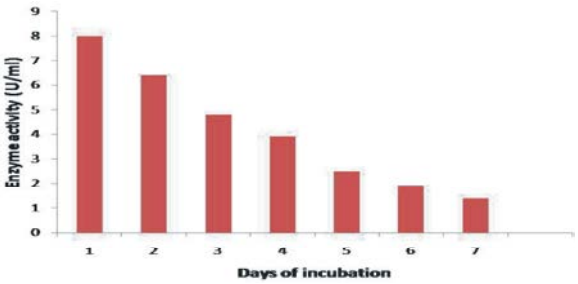

Fig. 4: Storage stability of catalase

DISCUSSION

The main aim of analyzing the phytochemical properties of *Nostoc* is to determine its biochemical composition. The extract of *Nostoc* contain alkaloids, saponin, steroids, flavonoids, phlobatannins, phenols and cynogenic glycoside, cardiac glycosides was absent. Protein content of *Nostoc* taken for analysis revealed that the presence of total protein about 3.03mg/ml. Catalase is wide spread in the nature which having been found in all aerobic organisms studied to date. Most of the work has been performed on the enzyme obtained from mammalian and bacterial sources where enzyme activity was present in high concentration. Previous studies had shown that catalase exist in multiple forms in several plants such as tobacco, saffron, cotton, mustard, maize, wheat, sunflower, castor bean, spinach, pepper, loblolly pine and kohlrabi [8].

Catalase activity of *Nostoc* was determined in the presence of hydrogen peroxide. The peak specific activity of catalase was 14.3 U/mg observed. There was rise in specific activity in each purification step[9]. The molecular weight of purified catalase of *Nostoc*was 34kDa. Molecular weight of *Saccharomyces cerevisiae* and *Escherichia coli* determined by gel filtration (66 kDa and 240 kDa) was in consonance with that obtained by SDS-PAGE reported [9, 10]. There is no previous report about catalase molecular weight from algae.

Catalase contains four porphyrinheme groups that allow the enzyme to react with the hydrogen peroxide [11]. Our study revealed the decomposition of hydrogen peroxide by catalase, the absorption decreased with time. The enzyme activity could be arrived at from this decrease.

A catalase was purified from the liver of bullfrog, Ranacatesbeiana Shaw, after extraction, ammonium sulfate precipitations, DEAE-Sephadex A-50 chromatography, gel filtration chromatography on a Sephadex G-150 column, ion exchange chromatography on a DEAE-Sephadex column and Sephacryl S-300 column. The yield and purification from the starting crude extract were 0.25% and 73.57-fold, respectively. The purified catalase with an apparent molecular mass of 186 kDa was shown to be composed of four identical subunits of apparent molecular mass of 47.7 kDa. The purified catalase is active over a broad pH range of 6.0–10.0 and it has an isoelectric point of 6.3. The enzyme showed a Km for H_2O_2 of 20 mM and an apparent Vmax of 51.91 U/mg and its maximum absorption was at 408 nm in the visible portion of the spectrum. In addition, the purified enzyme was markedly inhibited by azide, cyanide and hydroxylamine [12].

Since the separated enzyme appeared as a single band, it was concluded catalase enzyme to be tetrameric. From the reported data, it can be concluded that catalase varies in its MW depending upon the source of its isolation. Noted that the most interesting characteristics of catalase from *Nostoc*was the optimum pH and temperature. The optimum temperature and optimum pH for purified catalase from *Nostoc* on enzymatic reaction were 30°C and pH 7 respectively. *Archaeoglobusfulgidus,* the purification and characterization of catalase at various pH and found the similar pH characterization [13]. Similar temperature and pH has also been reported [14] on catalase enzyme of blood erythrocytes.

Catalase has been purified from *Agaricus bisporus* to homogeneity in the analytical ultracentrifuge, DEAE cellulose, Sephadox-75. Polyacrylamide gel electrophoresis revealed molecular weight 45kDa for catalase, which constituted 90% of the total protein of the stained gel, suggesting that the native enzyme is tetrameric [15].

The optimum temperature and pH for the purified enzyme catalase from *Pleurotus ostreatus* enzymatic reaction were 30°C and pH 7.5.Under denaturing conditions, polyacrylamide gel electrophoresis revealed dissociation of a major component of molecular weight 62 kDa [16].

CONCLUSION

After an investigation for several years, we found that *Nostoc* is also a promising plant to be applied in controlled ecological life support system many medicinal properties have been attributed to catalases including inhibition of platelet aggregation reduction of blood cholesterol concentrations prevention or alleviation of heart disease and reduction of blood glucose levels, also prevention or alleviation of infections caused by bacterial, viral, fungal and parasitic pathogens.

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